Trewick, 2005) indicate that the Acanthoxyla lineage most probably originated from one or few hybridization(s) between male Clitarchus hookeri (

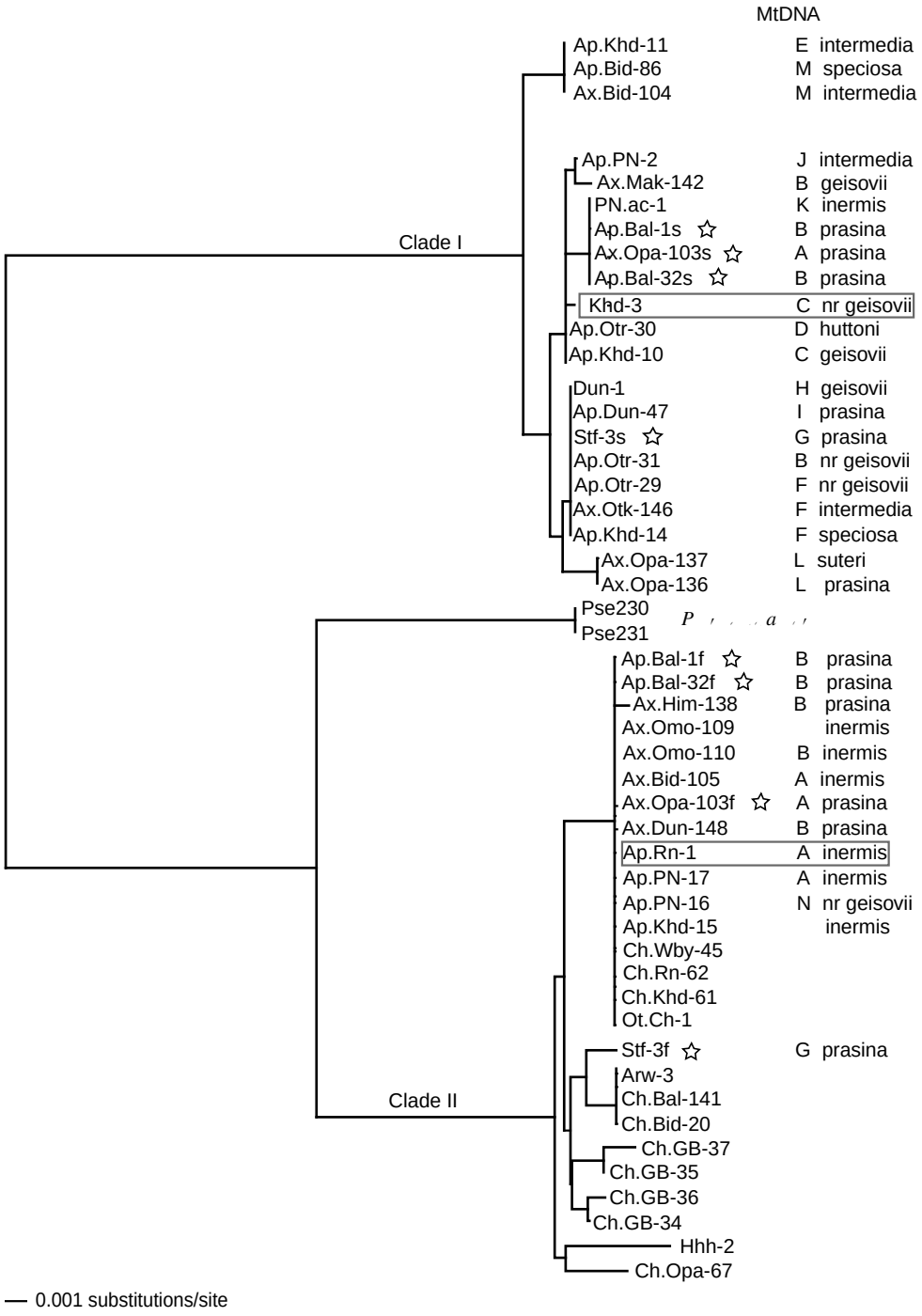

Fig. 1. Evidence from nuclear (rDNA) sequence that Clitarchus hookeri maybe one parent in hybrid origin of Acanthoxyla (Morgan-Richards and Trewick, 2005). Midpoint rooted Neighbor Joining (NJ) tree of ITS1&2 (internal transcribed spacers) nuclear DNA sequences comprising three clades: Aconthoyxla (Clade I), Pseudoclitarchus (Pse), and Clitarchus plus Acanthoxyla (Clade II). Acanthoxyla individuals are labelled with bold lettering, and annotated with species name (indicative of different morphologies) and COI–COII mtDNA haplotype (bold uppercase letter). Acanthoxyla individuals found to have both classes of ITS sequence are indicated by star, and the two individuals (Khd-3, Ap.Rn-1) used in other analyses presented here are indicated by grey boxes.

deeper taxonomic levels (Szymura et al., 1996; Paton and Baker, 2006) we have here further utilised the nuclear rDNA gene cluster. In their analysis of DNA sequences from stick insects and other insect orders where 28S represented a little less than 50% of the sequence data, Whiting et al. (2003) found that it provided some 62% of the phylogenetic signal and provided resolution throughout the tree topology. Hence our present analysis focuses on this gene, which provides a good chance of phylogenetic resolution, the opportunity to incorporate an existing extensive resource of data, and take advantage of the presence of both the maternal and paternal lineages in Acanothoxyla already identified by ITS sequences (Fig. 2).

2.2. Taxon sampling

Twenty-two species and nine genera of New Zealand stick insect are recognised by Jewell and Brock (2002). The genera (and number of species in brackets) for each subfamily are: Pachymorphinae—Micrarchus (1), Niveaphasma (1), Asteliaphasma (2), Tectarchus (4), Spinotectarchus (1); Phasmatinae—Acanthoxyla (8),

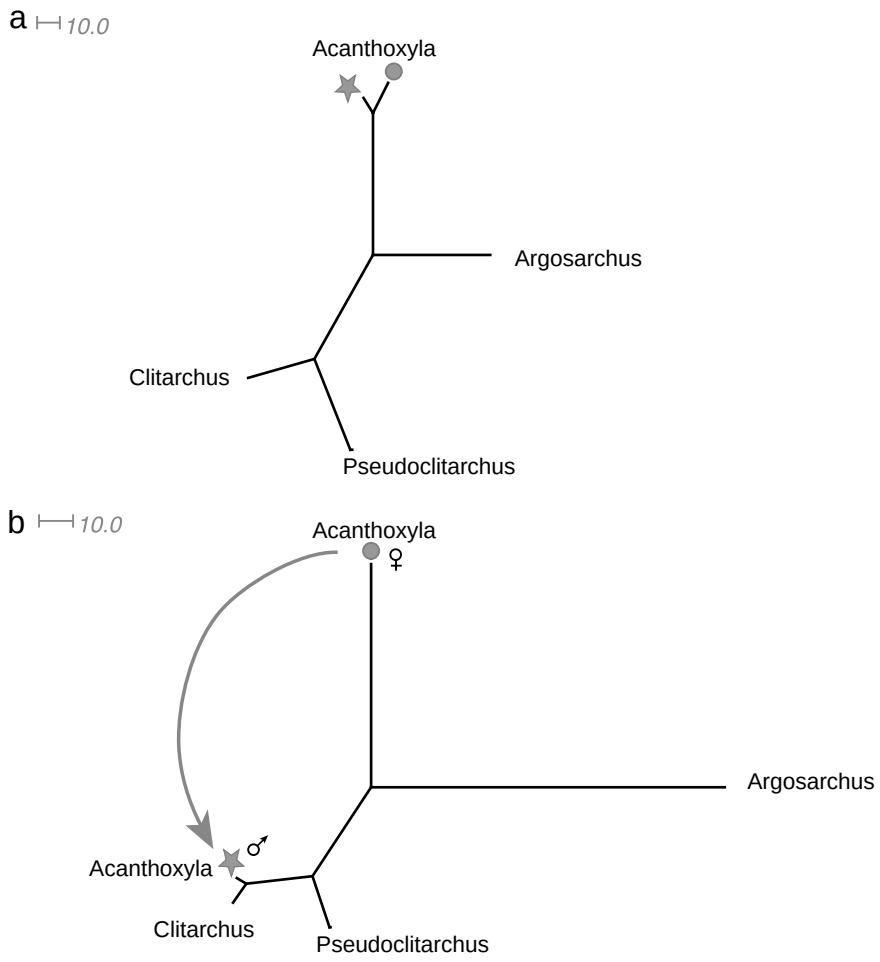

Fig. 2. Unrooted MP trees for (a) COI–COII mitochondrial, and (b) 28S and ITS nuclear DNA sequence data for representatives of the New Zealand "Phasmatinae". A single

Argosarchus (2), Clitarchus (2), Pseudoclitarchus (1). Of these, we have sampled 17 species for the present analysis, representing all genera (Appendix 1).

The species not included in the present study are unlikely to contribute to the current analysis, and comprise: (1) one species each from two genera (Clitarchus and Argosarchus) that were recently inferred from DNA sequence data to be monotypic (Trewick et al., 2005). (2) Despite several attempts, nobody has found Asteliaphasma naomi (Salmon, 1991) since its description from a single specimen. However, we have collected Asteliaphasma jucunda (Salmon, 1991) from the type location of A. naomi. A. naomi may have been an unusual morph of A. jucunda. (3) Three of the four Tectarchus (Salmon, 1948) species (T. huttoni (Brunner, 1907), T. ovobessus Salmon, 1948, T. salebrosus (Hutton, 1899)) are included but not the rare T. semilobatus (Salmon, 1948). (4) The present analyses include seven of the eight described species of Acanthoxyla (Fig. 1). Individuals representing two Acanthoxyla species (A. geisovii, A inermis) were selected for analysis of 28S as representing the extremes of COI–COII (1.8%) and ITS sequence diverge (Morgan-Richards and Trewick, 2005; see summary in Fig. 1).

We include additional geographic isolates of Micrarchus hystriculeus and Niveaphasma annulata (the latter including a putative new species identified by Tony Jewell) and the monotypic sexual species Pseudoclitarchus sentus Salmon, 1991. Pseudoclitarchus sentus is restricted to a single offshore island that is a New Zealand Scientific Reserve (Great King Island, Three Kings Islands) and as a consequence was previously not available for analysis (Morgan-Richards and Trewick, 2005). The inclusion of Pseudoclitarchus in the present analysis means that all New Zealand Phasmatinae (the subfamily that includes Acanthoxyla) are now represented. Although Salmon (1948) suggested Acanthoxyla was taxonomically near Clitarchus and Pseudclitarchus, Acanthoxyla species combine a suite of features including highly sculptured egg capsules, presence of abdominal flanges, well-developed thoracic spines and a distinctive opercular spine, and colours not seen in other stick insects in New Zealand.

Non-New Zealand stick insects were sampled using published sequences for a set of species reported by Whiting et al. (2003) and available from GenBank, with additional taxa from the West Pacific: Australia (3 species), New Caledonia (3 species), Fiji (2 species) and the Solomon Islands (2 species) (see Appendix). Identification of Pacific island and Australian stick insects was provided by Paul D. Brock (Natural History Museum, London) and Geoff Monteith (Brisbane Museum, Australia), respectively. Together these taxa comprise representatives of a range of subfamilies including those (Pachymorphinae, Phasmatinae) found in New Zealand (Appendix 1).

2.3. DNA extraction, amplification and sequencing

In most instances muscle tissue from fresh, frozen, or alcohol preserved specimens was removed from a leg for genomic DNA extraction using a salting-out method (Sunnucks and Hale, 1996). Tissue was macerated and incubated with 5 l L of 10 mg/mL

Proteinase-K in 600 l L of TNES buffer (20 mM EDTA, 50 mM Tris, 400 mM NaCl, 0.5% SDS) at 50 C for 1–4 h. 10% 5 M NaCl was added and the extractions shaken vigorously for 20 s followed by spinning at 14,000 rpm for 5 min. The supernatant was removed and precipitated with an equal volume of cold 100% ethanol. DNA was collected by spinning and washed with 70% ethanol, then dried and dissolved in water. In the case of pinned museum specimens (from Australia and New Caledonia), DNA extraction used incubation at 55 C with Proteinase-K and a CTAB buffer (2% Hexadecyltrimethylammonium bromide, 100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA), followed by a combined phenol/chloroform/isoamyl alcohol (25:24:1) cleanup.

2.4. PCR

To confirm the relationship and expected consistency of ITS and 28S sequences within Acanothoxyla we sequenced these genes plus the mitochondrial fragment comprising partial COI and COII genes from representatives of Acanthoxyla species (morphotypes) and the other New Zealand Phasmatinae (Argosarchus, Clitarchus, Pseudoclitarchus). We compiled COI–COII data from representatives of all New Zealand genera to assess the level of genetic diversity among them. All taxa were subjected to PCR and sequencing targeting the nuclear rDNA gene 28S.

The mitochondrial fragment, comprising the 3′ end of cytochrome oxidase I (COI), tRNA-Leucine, and cytochrome oxidase II (COII) was amplified using the primers C1-J-2195 and TK-N3785 (

Analysis of COI–COII sequence data was undertaken with tRNA Leucine excluded. Analysis of 28S DNA sequences used outgroup taxa selected by SeqSSi analysis that received a minimum of two

insects is inappropriate and/or (b) the information content of the mtDNA data at this level is negligible. The lowest intergeneric distance observed was between Pseudoclitarchus and Clitarchus (0.08); a relationship confirmed by phylogenetic analysis (Fig. 2a). Interspecific genetic distances from spatially separated samples were typical of those observed in New Zealand insects (0.02–0.05, Table 3).

The high functional constraint operating on the protein coding genes (COI–COII) imposes a major limitation on their phylogenetic utility and precludes their use for analysis of the relationships of stick insects at deeper taxonomic levels. Analysis of the 28S and ITS sequences together and apart revealed the same signal for two distinct nuclear lineages within Acanthoxyla (Fig. 2b). Given the close physical and thus heritable association of these two genes in the rDNA cluster this congruence is as expected. The addition of 28S sequence data confirms the previous observation of two (parental) lineages in Acanthoxyla (one close to Clitarchus hookeri), and the inclusion of new nuclear sequence data (28S and ITS) from Pseudoclitarchus sentus reveals that this species was not the source of either of these (Figs. 1 and 2).

SeqSSi results facilitated the selection of 11 putative outgroup/unidentified ingroup species (from a set of 44) with a ranked similarity score of A or B with at least one New Zealand stick insect species (Table 2). Examples of SeqSSi-plots are shown in Fig. 3. In these plots, two outgroups are plotted onto each graph to contrast the difference between sequences that were included in our analysis and those which were excluded. The 11 outgroup species selected with SeqSSi originated from Australia (5), Fiji (1), New Caledonia (1), New Guinea (1), South America (2) and the Caribbean (1). Analyses of 28S with New Zealand taxa and the 11 species selected by SeqSSi for the outgroup reveal two important features, (a) the New Zealand stick insects form a monophyletic clade in most cases (see below), and (b) Acanthoxyla forms a well-supported clade with Clitarchus and Pseudoclitarchus. The monophyly of the New Zealand taxa is supported by Bayesian analysis implemented by MrBayes and ML implemented by PAUP. In these analyses Spinotectarchus acornutus is placed as basal to the other New Zealand taxa. Bayesian posterior probabilities indicate strong support for this monophyletic grouping (Fig. 4), as does bootstrap resampling (10,000 replicates) using NJ distance criteria with a GTR+I+C model. To save computing time, a reduced data set of 12 taxa comprising representatives of the New Zealand fauna plus just 3 outgroup taxa, selected on the basis of previous analysis were subjected to ML (PAUP) bootstrap analysis (1000 replicates) employing a GTR+I+C

similarity). The value of SeqSSi is evident in enhanced phylogenetic resolution of the ingroup in analysis of the SeqSSi sample, and this probably reflects better alignments and enhanced model specification obtained from a more appropriate sample set.

The present data do not support monophyly of the subfamilies Phasmatinae and Pachymorphinae, neither within New Zealand nor across the geographic/taxonomic sample as a whole. The representatives of the two New Zealand subfamilies appear to be polyphyletic, including poor support for the grouping of Argosarchus with the other New Zealand Phasmatinae (Acanthoxyla, Clitarchus, Pseudoclitarchus). However, there is good evidence that the New Zealand fauna as a whole form a monophyletic group. The monotypic genus Spinotectarchus was consistently returned as sister to the other New Zealand taxa and as such these data support the classification of the genus Asteliaphasma Jewell and Brock (2002), splitting A. jucundus from S. acornutus; species that are clearly not sister taxa as implied by the previous classification (Salmon, 1991). Interestingly, the only New Zealand genus apart from Acanthoxyla with more than two described species in this study (Tectarchus) consistently failed to form a monophyletic clade, emphasising the inherent difficulty of resolving relationships within a group where morphology is extremely constrained for crypsis.

We cannot reject the hypothesis that the parthenogenetic genus Acanthoxyla is part of an exclusively New Zealand clade. We have included all described New Zealand genera and most species of New Zealand stick insects. It is possible that species remain undescribed, but it is less likely that genera remain unnoticed. Although two new New Zealand stick insect genera were recently erected, the species they comprise were already well characterised (Jewell and Brock, 2002). We have failed to find a mother for Acanthoxyla, but know where to look; New Zealand.

If a mother existed, we expected the unique Axanthoxyla ITS sequences of Clade I to be nested within its sexual maternal species in much the same way as the Acanthoxyla Clade II sequences are nested within Clitarchus hookeri diversity. In addition, mitochondrial DNA sequences from a maternal species would form part of the existing Acanthoxyla mtDNA clade. Salmon (1991) proposed, on the basis of morphological evidence that the genus ‘‘Pseudoclitarchus forms an evolutionary link between Clitarchus and Acanthoxyla”. Indeed, Pseudoclitarchus sentus was originally described by Salmon (1948) as an Acanthoxyla species (Acanthoxyla senta). Although a clade consisting of these three genera is supported by all DNA data, all analyses show that the sexual taxon Pseudoclitarchus sentus is not the maternal ancestor of Acanthoxyla. (

An inter-specific reproductive strategy of this type (hybridogenesis—Bullini, 1994) is known among Sicillian stick insects (Bacillus). In that case, successful egg production requires mating with males of the paternal species each generation but the paternal genome is discarded prior to gametogenesis, and the taxa involved (B. rossius-grandii) are congenerics (Mantovani and Scali, 1992; Mantovani et al., 2001). Karyological evidence for New Zealand stick insects indicates such a situation is unlikely (

analysis. The New Zealand Department of Conservation provided permits and we are particularly grateful for the assistance of Tony Beauchamp, Andrea Booth, Donna Stuthridge and Dave King. Specimens of Pseudoclitarchus sentus were collected on the Three King's Islands Nature Reserve by DoC staff under permit NO-13868-FAU,